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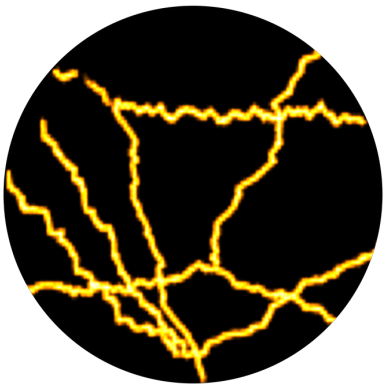
ThunderSTORM: a comprehensive ImageJ plugin for PALM and STORM data analysis and super-resolution imaging

User's Guide

Version 1.1



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1. About

1.1. Installation

ThunderSTORM is written in Java and is distributed under the terms of the GNU GPLv3 license as a plugin for ImageJ. The official download site for the latest version of ImageJ is accessible at http://rsbweb.nih.gov/ij/download.html. Note that ImageJ requires the Java Runtime Environment (JRE) to be installed. If you do not have JRE installed, download a bundled version of ImageJ & JRE for your platform. We recommend using the 64-bit version of Java for a 64-bit operating system.

To install the ThunderSTORM plugin for the first time, download the latest version from the website https://code.google.com/p/thunder-storm/ and copy the Java Archive file (Thunder_STORM.jar) into the plugins subfolder of your ImageJ installation. The location on a Windows system is, for example, "`C:\Program Files\ImageJ\plugins\Thunder_STORM.jar`".

To verify successful installation of ThunderSTORM, run ImageJ. There should be an item called `ThunderSTORM` in the `Plugins` menu of ImageJ. If the item does not exist, make sure that the file name `Thunder_STORM.jar` is correct as it has to contain the underscore "_" character.

1.2. Compatibility notes

- ThunderSTORM has been tested on Windows XP (32b), Windows 7 (32b/64b), Debian and Ubuntu Linux (32b/64b), and MacOS X 10.8.5.
- ThunderSTORM is compatible with ImageJ based applications such as Fiji and µManager.
- ThunderSTORM is compiled for Java 1.6 and is compatible with ImageJ 1.45s or later.

1.3. Help with ImageJ

The official ImageJ documentation is available at http://rsbweb.nih.gov/ij/docs/guide/user-guide.pdf.

1.4. Help with ThunderSTORM

To get help with using ThunderSTORM, please consult this User's manual or the project Wiki page. To get help related to a specific function, click on a blue icon with a question mark next to a particular control panel. A detailed description of algorithms used for the data analysis is also available in the Supplementary Note ThunderSTORM: Methodology and Algorithms. All documentation is accessible at https://code.google.com/p/thunder-storm/.

1.5. Initial settings of ImageJ

Since data acquired by Single Molecule Localization Microscopy (SMLM) are usually very large (several GB) and processing the data is computationally demanding, we recommend setting the number of processing threads equal to the number of available CPU cores. It is also crucial to set the memory limit to the highest possible value, but at the same time to keep enough memory (~2 GB) for the operating system.

For example, our setup uses a computer with an Intel i5 (4 cores) processor and 32 GB of RAM. We set the number of processing threads to 4 and the memory limit to 30 GB. To decrease memory consumption, load your data as a virtual stack (see ImageJ documentation).

To set the number of processing threads and the memory limit in ImageJ, select `Edit → Options → Memory & Threads`.

1.6. Updating ThunderSTORM

ThunderSTORM has an integrated updater. From ImageJ, select `Plugins → ThunderSTORM → Check for updates...` and select the version you want to upgrade (or downgrade) to, and click `OK`. If the updater cannot be used for some reason, download the latest `Thunder_STORM.jar` file from the project website, and copy it into the `plugins` subfolder of your ImageJ installation.

Please restart ImageJ before running the updated version of ThunderSTORM.

1.7. Input / Output

- Images – all image formats supported by ImageJ and its plugins.
- Molecular localizations and ground truth data – CSV, XSL, XML, YAML, JSON, and Tagged Spot File (TSF) format.

1.8. Building ThunderSTORM

To make any changes or upgrades to the code on your own, and to build ThunderSTORM, clone the Git repository by running the "`git clone https://code.google.com/p/thunder-storm/`" command from the Git console. The repository contains a NetBeans 7.4 project which can be compiled in the NetBeans IDE or from a console using the Ant utility.

In order to run ThunderSTORM directly from NetBeans, right-click on the `Thunder_STORM` project and select `Properties`. Then select `Run` and type the ImageJ path into the `Working directory` text field, for example, "`C:\Program Files\ImageJ`". Note that running ThunderSTORM directly from NetBeans will start ImageJ version 1.45s.

1.9. Report a bug

Prior to submitting a bug report, please upgrade to the latest version of ThunderSTORM and see if the error still occurs.

If you receive an exception that ImageJ ran out of memory, try to increase the memory limit or to load the SMLM data as a virtual stack (see ImageJ documentation).

In the case of a malfunction, please let us know by using the Google Code issue tracking system at the project website: https://code.google.com/p/thunder-storm/issues/list. Please specify the malfunction, and describe the procedure to reproduce the error. If an exception occurs, copy the stacktrace into the description box and specify your platform, i.e., your versions of Java, ImageJ, ThunderSTORM, and operating system.

2. Processing 2D data

This is an example of the workflow for processing 2D SMLM data in ThunderSTORM. Note that processing data with more channels should be done for each channel separately.

An example of (a fragment of) one of our real datasets is provided on the project website. More datasets, both artificial and real, can be found on the Single-Molecule Localization Microscopy Challenge website at http://bigwww.epfl.ch/smlm/.

Step 1 – Loading the data

Small datasets: Drag and drop the data file from the file explorer to ImageJ, or select `File → Open` in ImageJ, browse to the data file and press `Open`. This will load the entire image stack into memory.

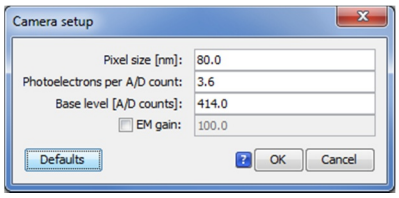
Large datasets: For datasets larger than the memory allocated to ImageJ, select `File → Import → TIFF Virtual Stack`, browse to the file and press `Open`. This loads just a TIFF header and the currently displayed frame into memory.

Sequence of images: If the dataset is not a single file but a sequence of files (all images have to be in the same folder), select `File → Import → Image Sequence`, browse to the first image of the sequence and press `Open`. The image sequence can also be opened with the Bio-Formats Importer plugin, which is available at http://www.openmicroscopy.org/site/products/bio-formats. Please be sure to use the latest version of Bio-Formats. For large datasets, use the `Virtual stack` option.

Step 2 – Camera settings

Set the camera pixel size in the sample plane, conversion factor between photons and digital units, base level offset of the camera digitizer, and EM gain of the camera. This is important for fitting methods and for estimation of localization accuracy, as these methods work with photon counts.

`Plugins → ThunderSTORM → Camera setup`



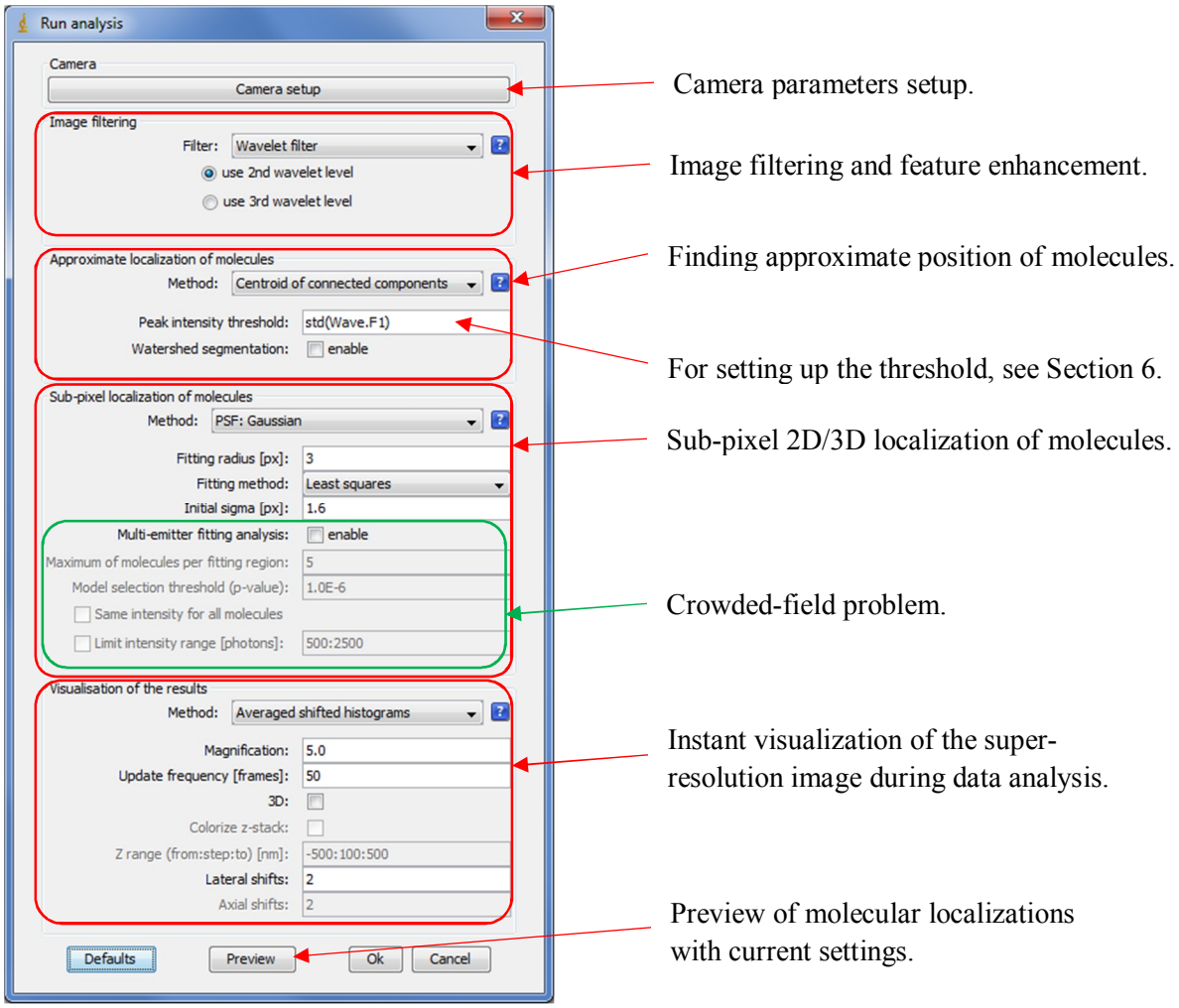
Step 3 – Processing the data

After opening the data, select `Plugins → ThunderSTORM → Run analysis`. There is a good deal of freedom for experienced users to set the processing parameters. However, the default options provide very good results on various datasets we have tried. Press `OK` to start the processing. An instant preview of the results achieved so far during the data analysis is shown. The table with results appears after the data analysis is finished.

Note that processing can be limited to an arbitrarily-shaped, user-specified region of interest in the input images. This can greatly speed up the processing in large data sets. The processing can be stopped at any point by pressing `Escape` on the keyboard.

Details about the algorithms used are accessible by clicking the particular help button, or in the Supplementary Note ThunderSTORM: Methodology and Algorithms.

`Plugins → ThunderSTORM → Run analysis`



Step 4 – Post-processing the data

The visualized super-resolution image and the table of localized molecules can be used as the final result. However, some of the fitted parameters might be estimated with poor quality due to noise in the input images, or for example, due to sample drift. Post-processing methods can be used to correct for this, see Section 4 for more information.

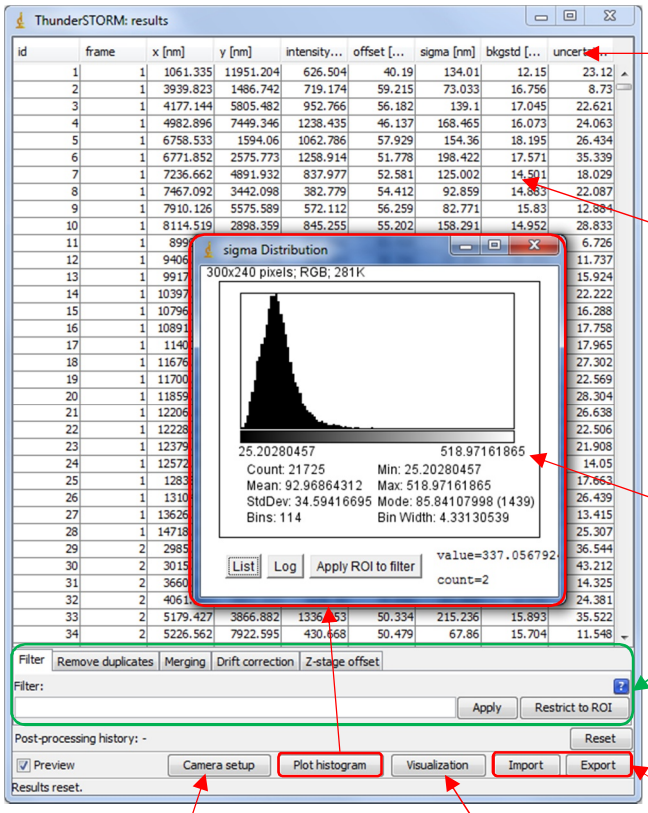
Step 5 – Visualization

Visualization involves creation of a new, high resolution image based on the previously obtained sub-diffraction molecular coordinates. Several methods have been implemented for both 2D and slice-by-slice 3D visualization. Choose one of them to visualize the data. Note that the table of results has to be open. Super-resolution images can be saved in any image format provided by ImageJ.

Step 6 – Importing / Exporting the table of results

ThunderSTORM can be used for post-processing and visualization of molecular localizations acquired also by other localization software. To allow easy interaction with other SMLM analysis packages, molecular localizations and ground truth data can be imported/exported to/from ThunderSTORM in various file formats, such as: CSV, XSL, XML, YAML, JSON, and Tagged Spot File (TSF) format.

`Plugins → ThunderSTORM → Import/Export → Show results table`



Left click to sort values.
Right click to change units.

Results of the analysis: localized molecules and their parameters. Select rows and right click on the selection to show the corresponding molecules in the overlay layer of the raw data, or to remove localizations in the selection by `Filter`, see Section 4.

Plotting the histogram of values for a particular variable can be used, e.g., to remove molecules with poor localization accuracy, see Section 4 for an example.

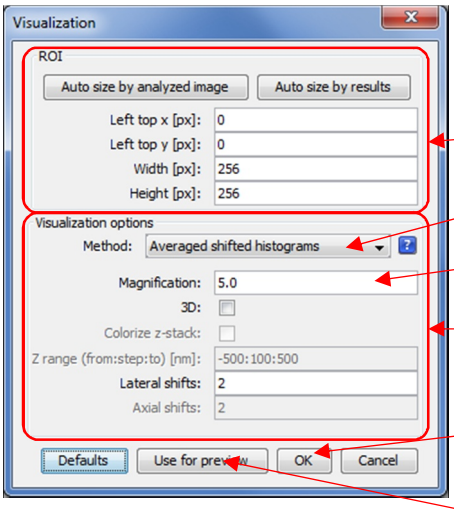
Post-processing methods, see Section 4.

Import/export of the results.

Camera parameters setup.

Visualization method for preview and final image.

`Plugins → ThunderSTORM → Visualization`



Defines camera ROI.

Visualization method.

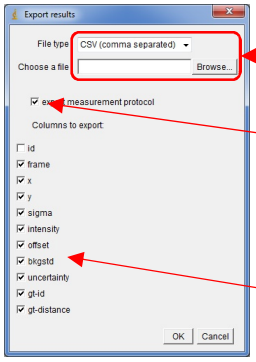
Magnification with respect to the raw image size.

Visualization options.

Create final super-resolution image.

Use selected options for the live preview.

`Plugins → ThunderSTORM → Import/Export → Export results`

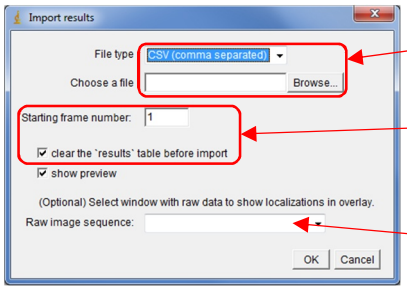


File name and data format.

Export data processing protocol in YAML format.

Measurements for export.

`Plugins → ThunderSTORM → Import/Export → Import results`



File name and data format.

Allows to concatenate several files with results into one table.

Show imported localizations as an overlay in the image sequence.

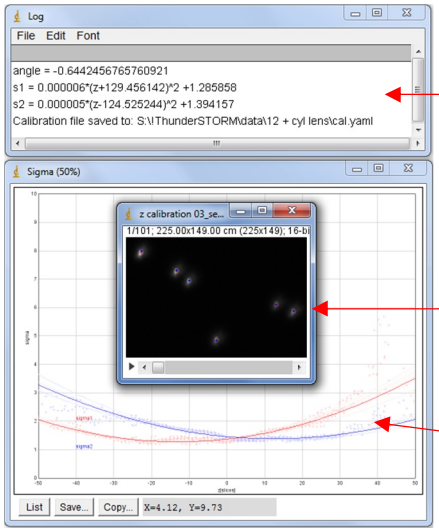
3. Processing 3D data (astigmatism method)

This is an example of the workflow for processing 3D SMLM data in ThunderSTORM in which the axial molecular position is estimated from astigmatism introduced by a cylindrical lens. The procedure is basically the same as in the case of processing 2D data. The only difference is that calibration of the system needs to be performed prior to processing the data in order to estimate the axial positions. An example of (a fragment of) one of our real 3D datasets is provided on the project website.

Step 1 – Calibration of the system

Load the calibration data and select `Plugins → ThunderSTORM → 3D calibration → Cylindrical lens calibration`. The default settings for processing the data should be fine to use. Specify the `Z stage step` (10 nm in our example dataset) and a location where the result of the calibration will be saved. Then click `OK` to run the calibration. All necessary information for 3D fitting is saved in the YAML file format.

Note that calibration can be limited to a user-specified region of interest in the input images.



Parameters of the fitted calibration curves and the angle of the astigmatic lens with respect to the camera.

Calibration z-stack, where beads used for calibration are marked with red circles and slice-by-slice sub-pixel localizations are marked with blue crosses.

Fitted calibration curves.

Step 2 – Processing the data

Load the image sequence acquired with the cylindrical lens and select `Plugins → ThunderSTORM → Run analysis`. In the panel `Sub-pixel localization of molecules`, select `PSF: Elliptical Gaussian (3D astigmatism)` and specify the path to the `Calibration file` generated in Step 1. Click `OK` to run the analysis.

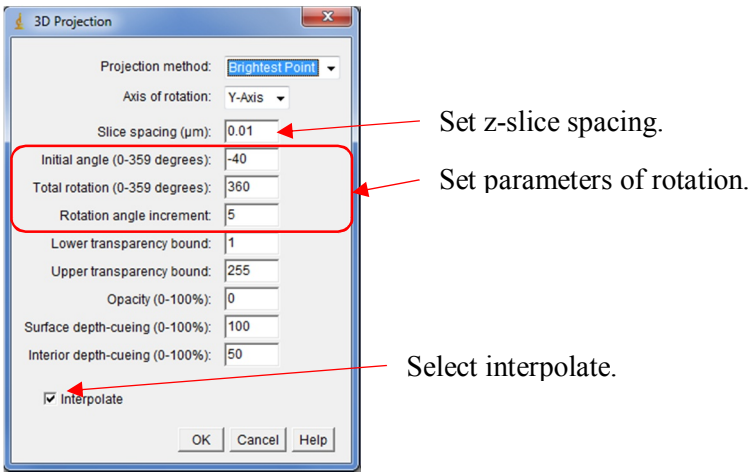
Step 3 – Visualization

Make sure that the `3D` option is enabled in the visualization dialog to get 3D super-resolution images, otherwise only a 2D image will be visualized. Specify the `Z range` to set the axial range and the step between slices. Enable the `Colorize z-stack` option to get a color-coded z-position (implemented as an ImageJ hyper-stack of channels with different lookup tables). Then Click `OK`.

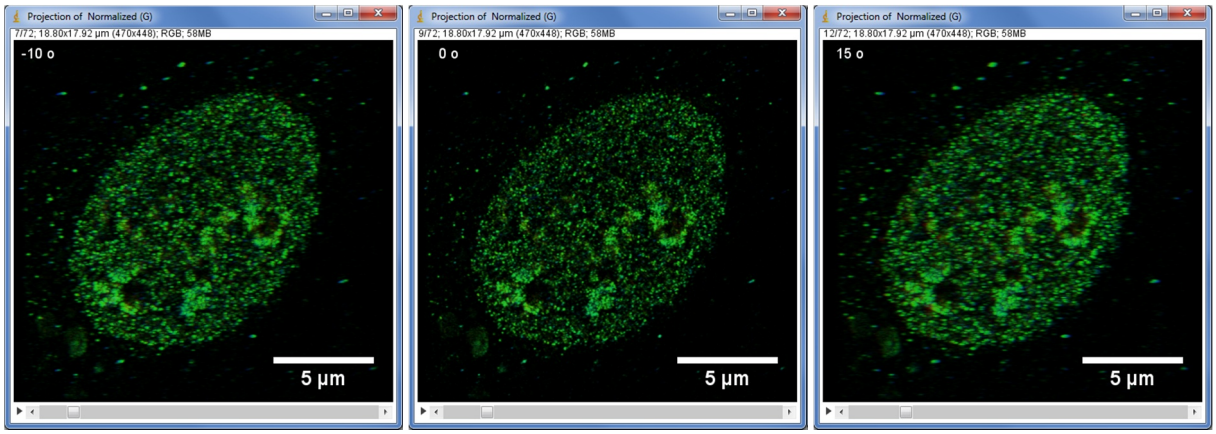
Step 4 – Visualizing a rotating 3D projection (optional)

A rotating 3D projection of the data can be generated using ImageJ feature such as `3D Project ...` The steps are as follows:

1) Create a slice-by-slice 3D visualization of the data.
2) Set optimal image contrast using the command `Image → Adjust → Brightness/Contrast (Ctrl+Shift+C)`. Press `Set` from the B&C menu to propagate the required minimum and maximum intensity value through the whole Z-stack.
3) Prior to creating a 3D projection, convert the image stack to 8-bits in the case of grayscale visualization, or to RGB, if the `Colorize z-stack` option was selected. Use commands `Image → Type → 8-bit` or `Image → Type → RGB Color`.
4) Set units and appropriate size of pixels for the final super-resolution image using the command: `Image → Properties ... (Ctrl+Shift+P)`.
5) A rotating 3D projection is created using the command `Image → Stacks → 3D Project ...`



6) Text with the rotation angle can be added: `Image → Stacks → Label ...` In the Label stack dialog, set the starting value, step interval, text location, and `Use Overaly`. The text color might need to be changed first using: `Image → Color → Color Picker ... (Ctrl+Shift+K)`.
7) To add a scale bar, use the command `Analyze → Tools → Scale Bar ...` Set scale bar length, text size, location, and select `Overlay`.



4. Post-processing modules

ThunderSTORM provides a set of post-processing methods to correct and optimize the results of the analysis. This step is optional but highly recommended. The order of processing steps is user-specified with a possibility to undo the last change or to restore the original values. All of the post-processing methods are described in detail in the Supplementary Note ThunderSTORM: Methodology and Algorithms.

4.1. Filter

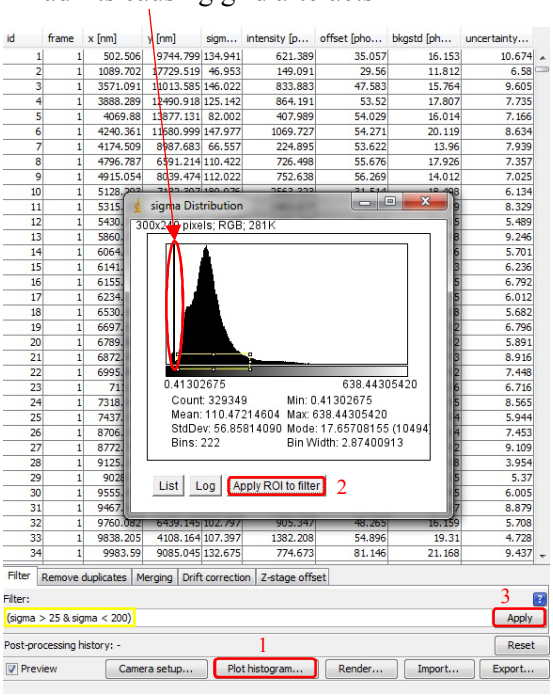
It is a common practice to remove molecules localized with poor precision, or with unrealistic parameters. The filtering criteria can be formulated in the `Filter` text field as an expression combining mathematical and logical functions and operators with parameters from the table of results obtained from previous data analysis.

The syntax and semantics rules for the formula interpreter are similar to the ones used in the threshold selection described in Section 6. The variables are scalars and vectors, where vectors are columns in the table of results specified by their names without units, e.g., `id`, `frame`, `x`, `y`, `sigma`, `intensity`, `offset`, `bkgstd`, `uncertainty`. The result of the formula must be a vector of booleans (true or false) for every molecule in the table. Only molecules with the condition equal to true are accepted. Built-in functions are the same as in Section 6, plus there are two logical functions "`a & b`" (AND) and "`a | b`" (OR).

Examples of filter expressions

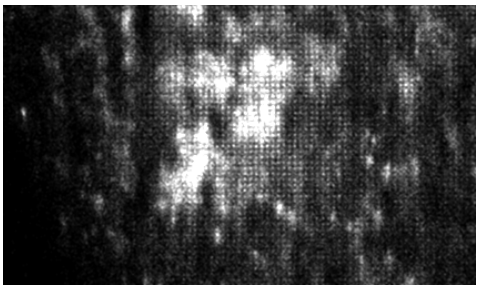
- `sigma>15`
- `frame<1000 | frame>2000`
- `intensity>100 & uncertainty<20`
- `(x-10000)^2+(y-10000)^2<2000^2`

Example of creating a filter expression using a histogram

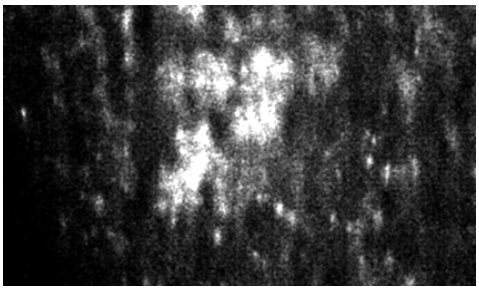


Bad fits causing grid artefacts

Super-resolution image with grid artefacts



Filtered super-resolution image



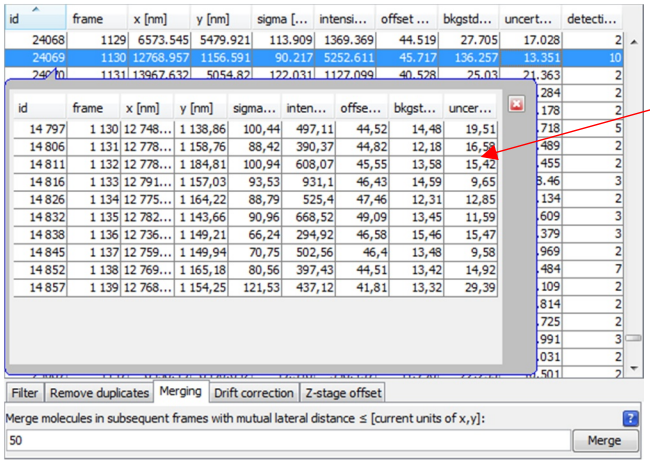
1) Select `Plot histogram` and select the parameter of choice (e.g., sigma).
2) Draw a rectangular selection in the histogram specifying the minimum and the maximum accepted value and press `Apply ROI to filter`. This inserts the formula into the `Filter` text field automatically.
3) Press `Apply` to apply the filtering rule and to update the image preview.

4.2. Remove duplicates

Repeated localizations of single molecules in one frame may occur due to overlapping fitting sub-regions when using multiple-emitter analysis approach. ThunderSTORM allows users to specify a radius below which molecules in close proximity are grouped together. Only the molecule with the smallest localization uncertainty in the group is kept, other molecules are removed. The radius can be specified as a mathematical expression which yields a scalar (same radius for all molecules, e.g., `5*mean(uncertainty)`) or a vector (different radius for every molecule, e.g., `3*uncertainty`).

4.3. Merging

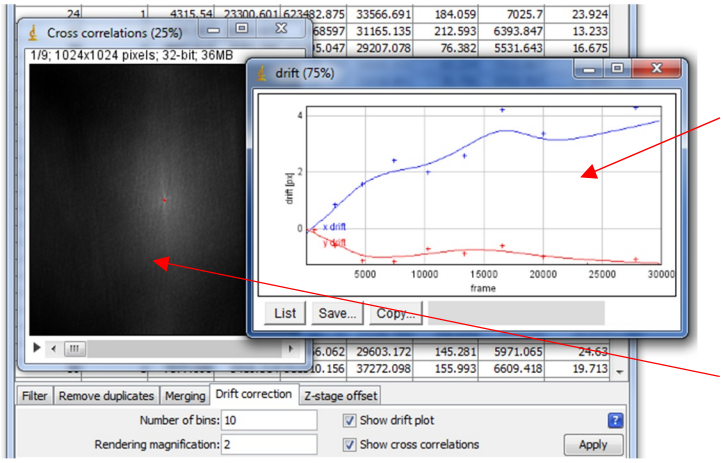
A single photo-activated molecule may appear in several sequential images. Such molecules can be combined into one single molecule based on a user-specified distance. After merging, a new column called `detections` appears in the table of results, showing the number of merged molecules. This new column can also be used for filtering.



Right-click on a particular row in the table of results and select `Show list of merged molecules` to see the list of molecules merged in that row.

4.4. Drift correction

ThunderSTORM supports lateral drift correction using the cross-correlation method.



Plot of lateral drift in time.

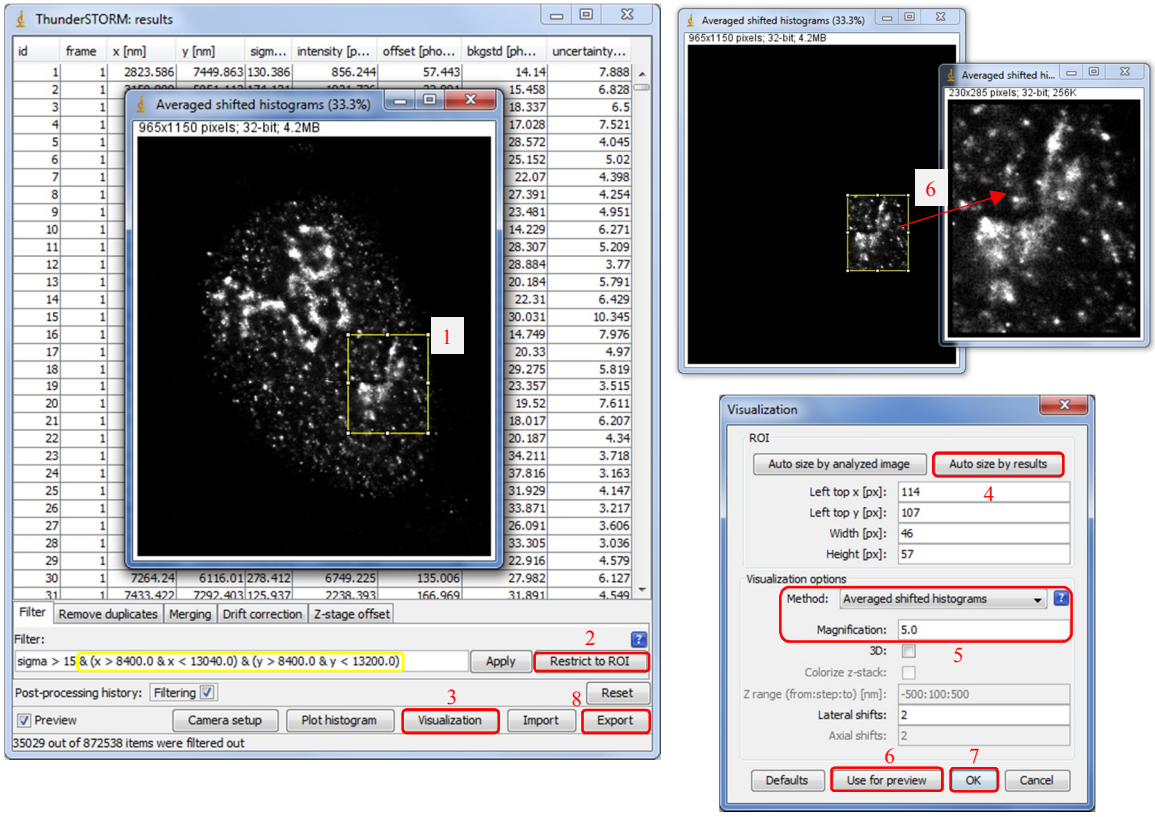
Cross-correlation image.

4.5. Z-stage offset

The axial field of view in 3D experiments can be extended by acquiring the data in multiple Z-stage positions. This method corrects the absolute axial positions of each molecule.

4.6. Visualizing and exporting molecular localizations from a region of interest

1) Select a rectangular region of interest (ROI) in the live preview image.
2) Press `Restrict to ROI`. A logical expression defining the ROI will be included in the `Filter`.
3) To resize the preview, or to visualize the data in the selected region, press `Visualization`.
4) Press `Auto size by results` to define the image coordinates based on ROI.
5) Set the visualization method and the desired magnification.
6) Press `Use for preview` to crop the live preview image based on ROI.
7) Or, press `OK` to visualize the final super-resolution image from the selected ROI.
8) To export data from the ROI, press `Export`.



5. Analyzing super-resolution images with ImageJ

Here we provide few examples of how ImageJ can be used for analyzing super-resolution images.

Setting image properties

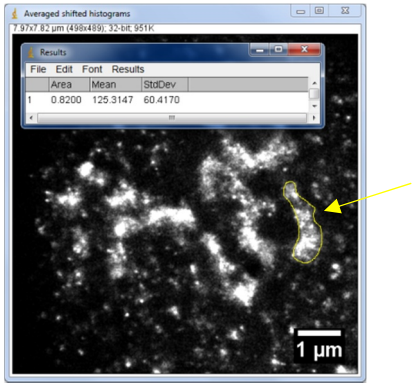
1) The first step is to set the units and pixel size in the super-resolution image using the command:
   `Image → Properties ... (Ctrl+Shift+P)`

Drawing a scale bar

1) A scale bar can be drawn using command:
   `Analyze → Tools → Scale Bar ...`

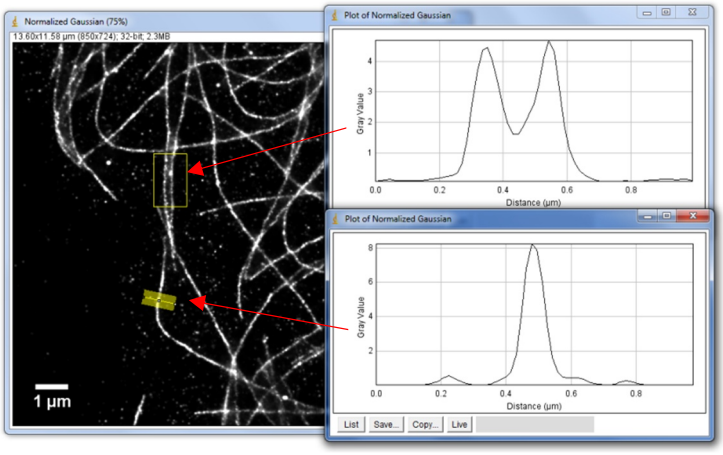
Measuring size of objects

1) Draw a region, e.g., free hand selection, around object of interest.
2) Measure the object size using command:
   `Analyze → Measure (Ctrl+M)`



Measuring an intensity profile

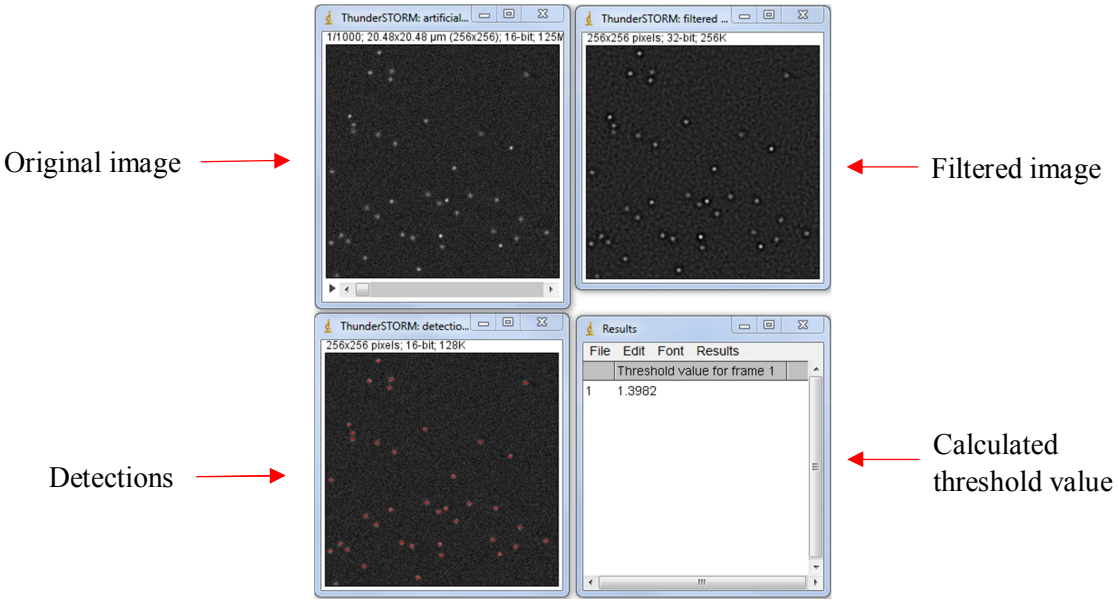
1) *Line selection*: set line width (double click on the line tool) and draw a line across the object of interest.
2) *Rectangular selection*: A rectangular selection can be used only for horizontal intensity profiles. Therefore, the image might need to be rotated first so that the structure of interest is vertical using command: `Image → Transform → Rotate ...`
3) The intensity profile can be plotted using the command `Analyze → Plot Profile (Ctrl+K).`



6. Specifying a threshold for detection of molecules

ThunderSTORM uses a single-valued intensity threshold which is updated for every input image. The threshold value can be specified by users as an expression combining mathematical functions and operators with variables based on the current raw or filtered image. This is a powerful option, because users can specify the threshold value systematically for unknown input images, where the global intensity may slowly fluctuate over time.

Use the `Preview` button in the `Run analysis` window to see which molecules are detected with the current settings. This can be useful for fine-tuning the threshold value given by the formula.



The formula interpreter provides several built-in statistical functions and some predefined variables. A brief description of the syntax and semantic rules follows.

Examples

- `150`
- `3*std(Wave.F1)`
- `0.2+min(F)`

Types of variables

- Scalar – a number
- Matrix – an image

Main syntax rules

- all names are case insensitive
- use a period (.) as a decimal point delimiter

Built-in operators

- `a + b` – addition
- `a - b` – subtraction
- `a * b` – multiplication
- `a / b` – division
- `a % b` – modulo
- `a ^ b` – b-th power of a

Semantics (all matrix operations are performed element-wise; matrices must have the same size)

- `scalar + scalar = scalar`
- `matrix + matrix = matrix`
- `matrix + scalar = matrix` (scalar is added to each element of the matrix)

Built-in functions

- `var(x)` – variance of x
- `std(x)` – standard deviation of x
- `mean(x)` – mean value of x
- `median(x)` – median of x
- `min(x)` – minimum in x
- `max(x)` – maximum in x
- `sum(x)` – sum of all items in x
- `abs(x)` – absolute value of x

Variables provided by different feature enhancement filters

All the filters provide these variables:

- `I` – input image without any changes
- `F` – final filtered image

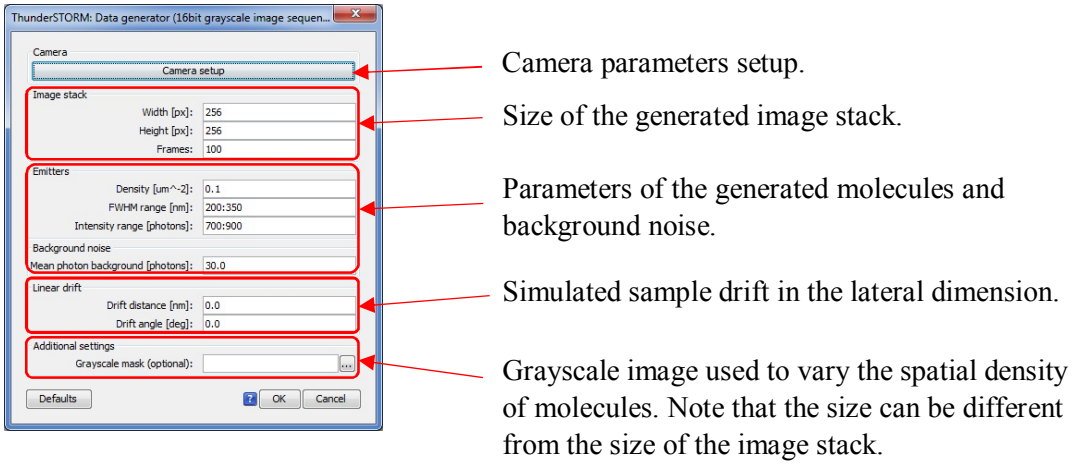
Variables representing the filters:

- `Box.F` – Averaging (Box) filter
- `Med.F` – Median filter
- `Gauss.F` – Gaussian filter
- `LowGauss` – Lowered Gaussian filter
- `DoG.F` – Difference-of-Gaussians filter
  - `DoG.G1` – Input image filtered by 1st Gaussian function of DoG filter
  - `DoG.G2` – Input image filtered by 2nd Gaussian function of DoG filter
- `DoB.F` – Difference of averaging filters
  - `DoB.B1` – Input image filtered by 1st Box of DoB filter
  - `DoB B2` – Input image filtered by 2nd Box of DoB filter
- `Wave.F` – Wavelet filter
  - `Wave.F1` – 1st Wavelet level of the input image
  - `Wave.F2` – 2nd Wavelet level of the input image
  - `Wave.F3` – 3rd Wavelet level of the input image

7. Generator of simulated data

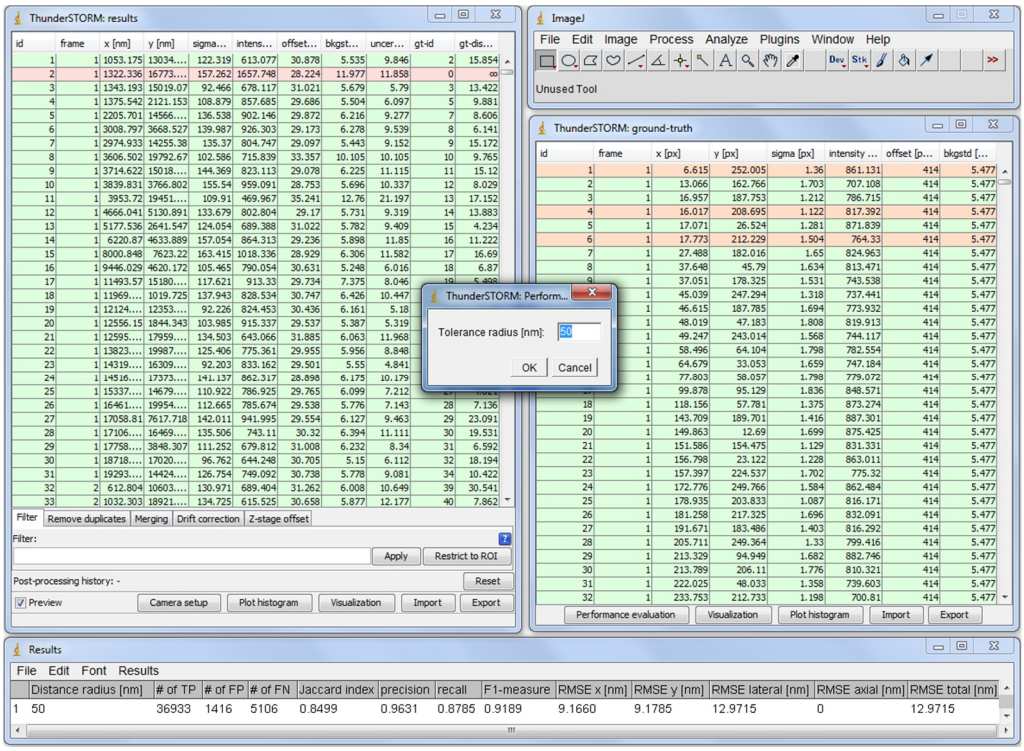
ThunderSTORM is capable of generating a sequence of SMLM-like images in which the ground-truth positions of the molecules are known. Users can specify the image size, sequence length, and ranges for the intensity, imaged size, and spatial density of the generated molecules. The spatial density of the molecules can be varied according to a user-supplied grayscale image.

`Plugins → ThunderSTORM → Performance testing → Generator of simulated data`


ThunderSTORM: Data generator (16bit grayscale image sequen...
Camera
Camera setup
Image stack
Width [px]: 256
Height [px]: 256
Frames: 100
Emitters
Density [um^-2]: 0.1
FWHM range [nm]: 200:350
Intensity range [photons]: 700:900
Background noise
Mean photon background [photons]: 30.0
Linear drift
Drift distance [nm]: 0.0
Drift angle [deg]: 0.0
Additional settings
Grayscale mask (optional):
Defaults
OK
Cancel
Camera parameters setup.
Size of the generated image stack.
Parameters of the generated molecules and background noise.
Simulated sample drift in the lateral dimension.
Grayscale image used to vary the spatial density of molecules. Note that the size can be different from the size of the image stack.

8. Performance evaluation

If ground truth molecular positions are available, ThunderSTORM can evaluate the performance of the processing methods. Ground truth data can be created by the generator of simulated data described in Section 7, or imported from a file by selecting `Plugins → ThunderSTORM → Import/Export → Import ground-truth`. To start evaluation, select `Plugins → ThunderSTORM → Performance testing → Performance evaluation`, enter the tolerance radius for accepting true-positive detections, and click `OK`. The results indicate true-positive detections (green), false positive detections (red), false negatives (orange), and related statistical measures (e.g., recall, precision, F1 score).

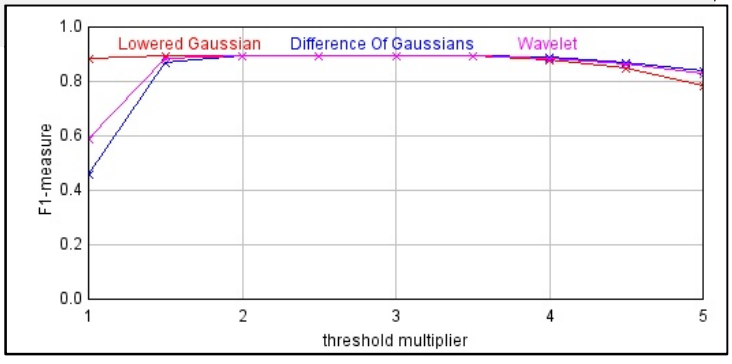


9. Monte Carlo simulations – an example using the ImageJ Macro Language

Any procedure in ThunderSTORM can be automated using the ImageJ Macro Language. Here we show an example of a Monte-Carlo simulation which searches for an optimal threshold value for different configurations of various localization algorithms. This example can be easily extended to any other combination of functions, such as automated sequential processing of several different datasets.

To get more information about the ImageJ Macro Language, see the official documentation at http://rsb.info.nih.gov/ij/developer/macro/macros.html.

```
requires("1.46c");      // because of `Array.concat` and `Array.slice` functions

run("Generator of simulated data", "width=256 height=256 frames=1000 density=0.3 "+
    "addpoissonvar=30.0 fwhmrange=200:350 intensityrange=700:900 driftdist=0.0 "+
    "driftangle=0.0");

filters = newArray("[Lowered Gaussian filter] sigma=1.6 size=11",
                   "[Difference-of-Gaussians filter] sigma1=1.3 sigma2=1.8 size=11",
                   "[Wavelet filter] level=[use 2nd wavelet level]");
colors = newArray("red","blue","magenta");
labelX = newArray(0.05, 0.33, 0.7);
labelText = newArray("Lowered Gaussian","Difference Of Gaussians","Wavelet");

xvals = newArray();
yvals = newArray();

len = 9;
fmax = 3;

for(f = 0; f < fmax; f += 1) {
    run("Clear Results");
    for(thr = 1.0, r = 0; thr <= 5.0; thr += 0.5, r += 1) {
        selectWindow("ThunderSTORM: artificial dataset");
        run("Run analysis", "filter="+filters[f]+
            " detector=[Local maximum] connectivity=8-neighbourhood threshold="+thr+
            "*std(F) estimator=[PSF: Integrated Gaussian] sigma=1.6 "+
            "method=[Least squares] fitradius=3 mfaenabled=false renderer=[No Renderer]");
        run("Performance evaluation","tolerance=50");
        setResult("Threshold multiplier",r,thr);
        xvals = Array.concat(xvals, thr);
        yvals = Array.concat(yvals,getResult("F1-measure",r));
    }
}

Plot.create("Simulation results", "threshold multiplier", "F1-measure");
Plot.setLimits(1.0,5.0,0.0,1.0);
for(f = 0; f < fmax; f += 1) {
    x = Array.slice(xvals,f*len,(f+1)*len);
    y = Array.slice(yvals,f*len,(f+1)*len);
    Plot.setColor(colors[f]);
    Plot.add("line",x,y);
    Plot.add("x",x,y);
    Plot.addText(labelText[f], labelX[f], 0.1);
}
Plot.show();
```



Note that results of the simulation can be further improved by using a more complex threshold for approximate molecular localizations, or by more complex post-processing rules.